Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for the Titration of Bovine Viral Diarrhea Virus in Vaccines

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Contact Person: Pat Foley (515) 663-7265
Peg Patterson (515) 663-7334

Approvals:

_____ Date:_____
Linn A. Wilbur, Head/Team Leader
Mammalian Virology Section

_____ Date:_____
Ann L. Wiegers, Quality Assurance Manager

_____ Date:_____
Randall L. Levings, Director
Center for Veterinary Biologics-Laboratory

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) is an *in vitro* assay method which employs a cell culture system utilizing cytopathic effect (CPE), direct fluorescence antibody (FA), and/or indirect FA (IFA) staining to determine the bovine viral diarrhea virus (BVDV) content of modified-live veterinary vaccines.

Note: For this SAM, the dilution terminology of 1:10, 1:20, etc. specifies 1 part plus 9 parts (liquid), 1 part plus 19 parts, etc.

1.2 Keywords

Bovine viral diarrhea virus; BVDV; potency test, titration, *in vitro*, CPE, FA, IFA

2. Materials

2.1 Equipment/instrumentation

2.1.1 Incubator,[1] 36° ± 2°C, high humidity, 5% ± 1% CO_2

2.1.2 Water bath,[2] 37° ± 1°C

2.1.3 Pipettors,[3] 25 µl, 500 µl, and tips[4]

2.1.4 Vortex mixer[5]

2.1.5 Multichannel pipettor, 50-300 µl x 8 or 12 channel[6]

[1] Model 3336, Forma Scientific, Inc., P.O. Box 649, Marietta, OH 45750-0649 or equivalent
[2] Model MW-1120A, Blue M Electric Co., 304 Hart St., Watertown, WI 53094 or equivalent
[3] Pipetman®, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent
[4] Cat. No. YE-3R, Analytic Lab Accessories, P.O. Box 345, Rockville Centre, NY 11571 or equivalent
[5] Vortex-3 Genie, Model G-560, Scientific Industries, Inc., Bohemia, NY 11716 or equivalent
[6] Finnipette®, Labsystems OY, Pulttitie 9, 00810 Helsinki 81, Finland or equivalent

2.1.6 Microscope,[7] inverted light

2.1.7 Ultraviolet light microscope[8]

2.2 Reagent/supplies

2.2.1 BVDV References[9]

2.2.1.1 NADL strain (cytopathic type I)

2.2.1.2 890 strain (noncytopathic type II)

2.2.1.3 125 strain (cytopathic type II)

2.2.2 Embryonic bovine kidney primary cells[10] (EBKp) found to be free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR).

2.2.3 Diluent Medium

2.2.3.1 9.61 g minimum essential medium with Earle's salts without bicarbonate[11]

2.2.3.2 2.2 g sodium bicarbonate ($NaHCO_3$)[12]

2.2.3.3 Dissolve with 900 ml deionized water (DW).

2.2.3.4 Add 5.0 g lactalbumin hydrolysate or edamine[13] to 10 ml DW. Heat to 60° ± 2°C until dissolved. Add to ingredients in **Section 2.2.3.3** with constant mixing.

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[7] Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747-3157 or equivalent
[8] Model BH2, Olympus America, Inc. or equivalent
[9] Reference quantities available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent
[10] Cat. No. ATCC CCL-22, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852-1776
[11] Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent
[12] Cat. No. S-5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent
[13] Edamine, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent

2.2.3.5 Q.S. to 1000 ml with DW; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[14]

2.2.3.6 Sterilize through a 0.22-µm filter.[15]

2.2.3.7 Aseptically add:

1. 10 ml L-glutamine[16]
2. 100 units/ml penicillin[17]
3. 50 µg/ml gentamicin sulfate[18]
4. 100 µg/ml streptomycin[19]
5. 2.5 µg/ml amphotericin B[20]

2.2.3.8 Store at 4° ± 2°C

2.2.4 Growth Medium

2.2.4.1 900 ml of Diluent Medium

2.2.4.2 Aseptically add 100 ml gamma-irradiated fetal bovine serum (FBS)

2.2.4.3 Store at 4° ± 2°C.

2.2.5 Anti-reference BVDV fluorescein isothiocyanate labeled conjugate[9] (Anti-reference BVDV Conjugate)

2.2.6 Monoclonal antibody (MAb) against BVDV type 1 or 2[9]

2.2.7 Anti-mouse fluorescein isothiocyanate labeled conjugate[21] (Anti-mouse Conjugate).

2.2.8 0.01 M Phosphate buffered saline (PBS)

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[14] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent
[15] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent
[16] L-glutamine-200 mM (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent
[17] Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent
[18] Cat. No. 0061-0464-04, Schering Laboratories or equivalent
[19] Cat. No. S 9137, Sigma Chemical Co. or equivalent
[20] Cat. No. A 4888, Sigma Chemical Co. or equivalent
[21] Cat. No. 04-6111, Zymed Laboratories, Inc., 458 Carlton Ct., So. San Francisco, CA 94080 or equivalent

2.2.8.1 1.19 g sodium phosphate, dibasic, anhydrous[22] (Na_2HPO_4)

2.2.8.2 0.22 g sodium phosphate, monobasic, monohydrate[23] ($NaH_2PO_4 \cdot H_2O$)

2.2.8.3 8.5 g sodium chloride[24] (NaCl)

2.2.8.4 Q.S. to 1000 ml with DW

2.2.8.5 Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide[25] (NaOH) or 2N HCl

2.2.8.6 Sterilize by autoclaving at 15 psi, 121° ± 2°C for 35 ± 5 min; store at 4° ± 2°C.

2.2.9 Trypsin versene (TV) Solution

2.2.9.1 8.0 g NaCl

2.2.9.2 0.40 g KCl

2.2.9.3 0.58 g $NaHCO_3$

2.2.9.4 0.50 g irradiated trypsin[26]

2.2.9.5 0.20 g versene or disodium salt ethylenediaminetetraacetic acid (EDTA)[27]

2.2.9.6 1.0 g dextrose

2.2.9.7 0.4 ml 0.5% phenol red[28]

2.2.9.8 Q.S. with DW to 1000 ml.

2.2.9.9 pH to 7.3 with $NaHCO_3$.

[22] Cat. No. S 0876, Sigma Chemical Co. or equivalent
[23] Cat. No. S 9638, Sigma Chemical Co. or equivalent
[24] Cat. No. S 9625, Sigma Chemical Co. or equivalent
[25] Cat. No. 925-30, Sigma Chemical Co. or equivalent
[26] Cat. No. 0152-15-9, DIFCO Laboratories, P.O. Box 331058, Detroit, MI 48232-0758
[27] Cat. No. E 5134, Sigma Chemical Co. or equivalent
[28] Cat. No. P 0290, Sigma Chemical Co. or equivalent

2.2.9.10 Filter through a 0.22-µm filter.

2.2.9.11 Store at -20° ± 4°C.

2.2.10 80% Acetone

2.2.10.1 80 ml acetone[28]

2.2.10.2 20 ml distilled water

2.2.10.3 Store at room temperature (RT) (23° ± 2°C).

2.2.11 Tissue culture plates, 96 well[29]

2.2.12 Polystyrene tubes, 17 x 100 mm[30]

2.2.13 Polystyrene tubes, 12 x 75 mm[31]

2.2.14 Serological pipette, 10 ml[32]

2.2.15 Graduated cylinders, 25 ml, 50 ml, 100 ml, and 250 ml,[33] sterile

2.2.16 Infectious bovine rhinotracheitis monospecific antiserum[9] (IBR AS)

2.2.17 Parainfluenza 3 virus monospecific antiserum[9] (PI3V AS)

2.2.18 Bovine respiratory syncytial virus monospecific antiserum[9] (BRSV AS)

2.2.19 Plastic squirt bottle, 500 ml[34]

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[29] Cat. No. 3596, Corning Costar Corp., 1 Alewife Center, Cambridge, MA 02140 or equivalent
[30] Falcon® 2057, Becton Dickinson Labware, 2 Bridgewater Lane, Lincoln Park, NJ 07035 or equivalent
[31] Falcon® 2058, Becton Dickinson Labware or equivalent
[32] Falcon® 7530, Becton Dickinson Labware or equivalent
[33] Cat. No. P34546-02, P34546-03, P34546-04, and P34546-05 respectively, Cole-Parmer Instrument Co., 625 East Bunker Court, Vernon Hills, IL 60061-9872 or equivalent
[34] Cat. No. 2402, Nalge Nunc Int., 75 Panorama Creek Dr., Rochester, NY 14602 or equivalent

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training in cell culture technique, the principles of aseptic technique, and virus titration assays.

3.2 Preparation of equipment/instrumentation

On the day of test initiation, set the water bath at 36° ± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of EBKp Plates.

3.3.1.1 Cells are prepared from healthy, confluent EBKp (pass 3 or lower) cells, that are maintained by passing every 5 ± 2 days. One day prior to test initiation, cells are removed from the growth containers by using TV Solution. Using a multichannel pipettor, add 200 µl/well of $10^{5.4}$ to $10^{5.6}$ cells/ml cells suspended in Growth Medium into all wells of a 96-well cell culture plate. Prepare 1 EBKp plate for the controls and the first Test Serial. Each additional plate allows testing of 2 Test Serials. These become the EBKp Plates. Incubate at 36° ± 2°C in a CO_2 incubator for 24 ± 12 hr. Growth Medium is not changed unless excess acidity occurs or cells are not confluent in 24 hr.

3.3.2 Preparation of BVDV Reference Control.

3.3.2.1 On the day of test initiation, the BVDV Reference Control is selected based on the genotype (type I or II) and the phenotype (cytopathic or noncytopathic) of the vaccine virus to be titered. Once selected, a vial of the BVDV Reference is rapidly thawed in a 36° ± 2°C water bath and tenfold dilutions made:

1. Place 4.5 ml of Diluent Medium into 6, 17 x 100-mm polystyrene tubes labeled 10^{-1} to 10^{-6} respectively, using a 10-ml serological pipette.

2. Using a 500 µl pipettor, transfer 500 µl of BVDV Reference to the 10^{-1} tube; mix by vortexing. Discard pipette tip.

3. Using a new pipette tip, transfer 500 µl from the 10^{-1} labeled tube to the 10^{-2} tube; mix by vortexing.

4. Repeat **Section 3.3.2.1.3** for each of the subsequent dilutions, transferring 500 µl from the previous dilution to the next dilution tube until the dilution series is completed.

3.3.3 Working Anti-reference BVDV Conjugate. On the day of EBKp Plate examination, if an FA test is to be conducted, dilute the appropriate Anti-reference BVDV Conjugate in PBS, according to the CVB-L supplied Reference and Reagent Sheet or as determined for that specific conjugate.

3.3.4 Working Anti-BVDV MAb. On the day of EBKp Plate examination, if an IFA test is to be conducted, dilute the appropriate Anti-BVDV MAb in PBS, according to the CVB-L supplied Reference and Reagent Sheet or as determined for that specific MAb.

3.3.5 Working Anti-mouse Conjugate. On the day of EBKp Plate examination, if an IFA test is to be conducted, dilute the Anti-mouse Conjugate in PBS, according to the manufacturer’s recommendations.

3.4 Preparation of the sample

3.4.1 The initial test of a Test Serial will be with a single vial (a single sample from 1 vial). On the day of test initiation, remove the seal and stopper from both the Test Serial bottle and the bottle containing the accompanying diluent. Measure the diluent into a sterile graduated cylinder according to the number of doses indicated on the manufacturer's instructions (e.g. for 50 dose container of 2 ml per dose, reconstitute with 100 ml of diluent) and aseptically pour the diluent into the lyophilized bottle of vaccine. Mix by vortexing.

3.4.2 Viral neutralization. In order to determine the BVDV titer in a multifraction product, on the day of test initiation, neutralize the IBR, PI3V, and BRSV fractions with monospecific antiserum.

3.4.2.1 IBR/BVDV Vaccine

1. 1.0 ml of the reconstituted Test Serial is diluted with 4.0 ml of Diluent Medium in a 17 x 100-mm polystyrene tube; mix by vortexing.

2. Mix 500 µl of **Section 3.4.2.1.1** dilution of the Test Serial with 500 µl IBR AS in a 12 x 75-mm polystyrene tube, labeled 10^{-1}. Mix by vortexing.

3. Incubate at room temperature (RT) (23° ± 2°C) for 45 ± 15 min.

4. The mixture constitutes a 10^{-1} dilution of the Test Serial.

3.4.2.2 IBR/PI3V/BVDV Vaccine

1. 1.0 ml of the reconstituted Test Serial is diluted with 4.0 ml of Diluent Medium in a 17 x 100-mm polystyrene tube; mix by vortexing.

2. 1.0 ml of **Section 3.4.2.2.1** dilution of the Test Serial is mixed with 500 µl of IBR AS and 500 µl of PI3V AS in a 12 x 75-mm polystyrene tube, labeled 10^{-1}. Mix by vortexing

3. Incubate at RT for 45 ± 15 min.

4. The mixture constitutes a 10^{-1} dilution of the Test Serial.

3.4.2.3 IBR/PI3V/BVD/BRSV Vaccine

1. 1.0 ml of the reconstituted Test Serial is diluted with 4.0 ml of Diluent Medium in a 17 x 100-mm polystyrene tube; mix by vortexing.

2. 1.5 ml of **Section 3.4.2.3.1** dilution of the Test Serial is mixed with 500 µl of IBR AS, 500 µl of PI3V AS and 500 µl of BRSV AS in a 12 x 75-mm polystyrene tube, labeled 10^{-1}. Mix by vortexing.

3. Incubate at RT for 45 ± 15 min.

4. The mixture constitutes a 10^{-1} dilution of the Test Serial.

3.4.2.4 BVDV Monovalent Vaccine

1. 500 µl of the reconstituted Test Serial is diluted with 4.5 ml of Diluent Medium in a 17 x 100-mm polystyrene tube, labeled 10^{-1}. Mix by vortexing.

2. The mixture constitutes a 10^{-1} dilution of the Test Serial.

3.4.3 Sample dilutions. Five, tenfold dilutions are made from the 10^{-1} dilution of the Test Serial using Diluent Medium.

1. Place 4.5 ml of Diluent Medium into each of five 17 x 100-mm polystyrene tubes labeled 10^{-2} through 10^{-6}, using a 10-ml serological pipette.

2. Pipet 500 µl of the Test Serial from the 10^{-1} tube into the 10^{-2} tube, using a 500 µl pipettor; mix by vortexing. Discard pipette tip.

3. Using a new tip each time repeat **Section 3.4.3.2** to the remaining tubes transferring 500 µl from the previous dilution tube to the next tube until the final dilution is made (10^{-6}); mix by vortexing between each dilution.

4. Performance of the test

4.1 On the day of test initiation, inoculate 5 wells/dilution with 50 µl/well of the diluted Test Serial and the BVDV Reference Control (10^{-3} through 10^{-6}). Change tips between each unique sample (e.g., each Test Serial and the BVDV Reference Control), but tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g. 10^{-6} through 10^{-3}). This becomes the EBKp Test Plate.

4.2 Maintain 5 wells as uninoculated cell culture controls on each plate.

4.3 Incubate the EBKp Test Plate undisturbed at 36° ± 2°C in a CO_2 incubator for 96 ± 6 hr.

4.4 At the end of incubation, examine the EBKp Test Plate at 100X magnification on an inverted light microscope for CPE characterized by cell fusion.

4.4.1 Wells displaying 1 or more CPE foci, are considered to be positive for BVDV.

4.4.2 Results are recorded as the number of CPE positive wells versus the total number of wells examined for each dilution of the Test Serial and the BVDV Reference Control.

4.5 If CPE is not detected by microscopic examination, the EBKp Test Plate may be read by FA or IFA (FA used for most type 1 BVDV; IFA used for type 2 BVDV):

4.5.1 FA method:

1. Growth Medium is decanted from the EBKp Test Plate into a suitable autoclavable container and cells are rinsed once with RT PBS using a plastic wash bottle or by immersion into a pan filled with RT PBS. PBS is decanted immediately after filling.

2. Fill each well of the EBKp Test Plate with 80% Acetone using a plastic squirt bottle.

3. The EBKp Test Plate is allowed to incubate for 15 ± 5 min at RT.

4. Decant the 80% Acetone into a suitable container.

5. Using a multichannel pipettor add 200 µl/well of the Anti-reference BVDV Conjugate to each well of the EBKp Test Plate.

6. The EBKp Test Plate is incubated for 30 ± 15 min at 36° ± 2°C.

7. Decant the conjugate from all wells into suitable container and rinse twice with PBS as in **Section 4.5.1.1**. The Test Plate may be read immediately or air dried.

8. The monolayers are examined for specific cytoplasmic fluorescence using an ultraviolet light microscope at 100X magnification. Wells containing one or more FA foci clusters adhering to the cell monolayer are considered to be positive for BVDV.

9. Record the number of FA positive wells versus the total number of wells examined for each dilution of the Test Serial and the BVDV Reference Control.

4.5.2 IFA method:

1. Proceed as for FA procedure in **Section 4.5.1.1 through 4.5.1.4.**

2. Using a multichannel pipettor, add 200 µl/well of the MAb against BVDV type 1 or 2, depending on which type is to be titrated, to all wells of the EBKp Test Plate.

3. The EBKp Test Plate is incubated for 30 ± 15 min at 36° ± 2°C.

4. Decant the monoclonal antibody from wells and rinse 2 X with PBS. Remove as much PBS as possible by blotting on an absorbent surface.

5. Using a multichannel pipettor add 200 µl/well of Anti-mouse Conjugate to all wells of the EBKp Test Plate.

6. The EBKp Test Plate is incubated for 30 ± 15 min at 36° ± 2°C.

7. Proceed as in **Section 4.5.1.7 through 4.5.1.9**.

4.6 Calculate the BVDV endpoints of the Test Serial and the BVDV Reference Control using the method of Spearman-Kärber as commonly modified. The titers are expressed as $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$) of the test wells.

Example:

10^{-3} dilution of Test Serial = 5/5 wells CPE/FA positive
10^{-4} dilution of Test Serial = 5/5 wells CPE/FA positive
10^{-5} dilution of Test Serial = 2/5 wells CPE/FA positive
10^{-6} dilution of Test Serial = 0/5 wells CPE/FA positive

Test dose titer = (X - d/2 + [d * S])
where:

X = $\log_{10}$ of lowest dilution (3)
d = $\log_{10}$ of dilution factor (1)
S = sum of proportion of CPE/FA positive

$$\frac{(5 + 5 + 2)}{5} = \frac{12}{5} = 2.4$$

Test dose titer = (3 - 1/2 + (1 * 2.4) = 4.9

Adjust the titer to the Test Serial dose size by adding the $\log_{10}$ of the reciprocal of the Inoculation Dose divided by the Test Serial Dose where:

Inoculation Dose = amount of diluted Test Serial added to each well of the Test Plate

Test Serial Dose = Manufacturer’s recommended vaccination dose

Example:

BVDV endpoint = 4.9

$$\frac{0.025 \text{ ml inoculum}}{2 \text{ ml dose}} = \frac{1}{80} = 1.9 \log$$

Total = 6.8 log

Titer of the Test Serial is $10^{6.8}$ $TCID_{50}$.

5. Interpretation of the test results

5.1 For a valid assay

5.1.1 The calculated $TCID_{50}$ titer of the BVDV Reference Control must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.1.2 The uninoculated cell controls can not exhibit any CPE or cloudy media that would indicate contamination.

5.1.3 The lowest dilution of the BVDV Reference Control must exhibit a 100% positive CPE/FA (5/5), and the highest (most dilute) must exhibit no positive CPE/FA (0/5).

5.2 If the validity requirements are not met, then the assay is considered a **NO TEST** and may be retested without prejudice.

5.3 If the validity requirements are met and the titer of the Test Serial is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production, the Test Serial is considered **SATISFACTORY**.

5.4 If the validity requirements are met but the titer of the Test Serial is less than the titer contained in the APHIS filed Outline of Production, the Test Serial is retested according to 9 CFR, Part 113.8.b.

6. Report of test results

6.1 Results are reported as $TCID_{50}$ per dose.

6.2 Record all test results on the test record.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.309, U.S. Government Printing Office, Washington, DC, 2000.

7.2 Cottral GE, (Ed.), 1978, *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, NY, pg.731.

7.3 Finney, DJ, 1978, *Statistical method in biological assay*. Griffin, London. 3rd edition, pg.508.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, to provide additional detail and to reflect these changes from the superseded protocol:

1) replacement of the 8 chamber glass slides by 96-well plates,

2) inclusion of newly added vaccine viruses to the serum neutralization step, and

3) inclusion of IFA for detection of type 2 BVDV.